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16. ABSTRACT

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SUBCHRONIC TOXICOLOGY OF HUMIC ACID FOLLOWING CHLORINATION IN THE RAT

Lyman W. Condie, R. Dana Laurie, J. Peter Bercz

Target Organ Toxicology Branch, Toxicology and Microbiology Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio

A subchronic 90-d study was conducted with chlorinated and nonchlorinated humic acids using male Sprague-Dawley rats. Body weight gain, terminal organ and body weights, food and fluid consumption, clinical chemistries, hematological parameters, and urinalyses were determined for all animals. Selected organs were examined microscopially. Significant findings were confined to those rats given the high dose of chlorinated humic acid (1.0 g/l total organic carbon). The terminal body weight and average weekly body weight gain were significantly lower ($p < 0.05$) in the high-dose group as compared to the distilled-water control group. This difference can be partially explained by a 16% lower daily fluid consumption. The average weight of the kidneys was significantly higher in the 1.0-g/l chlorinated humic group as compared to distilled-water controls. Hematological parameters and clinical chemistry values were normal in all treatment groups. The most significant finding was the increased incidence and severity of hematuria in the 1.0-g/l chlorinated humic acid group. A thorough histopathological examination of the entire urinary tract indicated that the most likely cause of the more severe incidences of hematuria in the rats was caused by crystalline deposits in the renal pelvis.

INTRODUCTION

Naturally occurring humic and fulvic acids comprise the bulk of organic material which is present in most surface waters. Considerable attention was placed on aquatic humic substances following the discovery that the presence of trihalomethanes in drinking water was due to the reaction of chlorine with natural substances, presumably humic material (Rook, 1974). Investigators subsequently confirmed that chlorination of humic or fulvic acids results in trihalomethane formation (Rook, 1976; Stevens et al., 1978). Additional chlorinated and nonchlorinated organic products have been demonstrated to be formed as a result of aqueous

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Requests for reprints should be sent to Lyman W. Condie, Target Organ Toxicology Branch, Toxicology and Microbiology Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268.

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chlorination of humic materials (Rook, 1977; Christman et al., 1980), which suggests that humic materials may serve as precursors to many of the organic chemicals formed during the chlorination of drinking water.

The organic compounds that have been identified in drinking water are generally volatile organics and account for only 5-10% of the total organic content of drinking water (National Academy of Sciences, 1977). Little is known about the identity of the non-volatile organic compounds in drinking water because they have been difficult to identify with state-of-the-art techniques. Since the majority of drinking water contaminants have not been identified, the health risks resulting from the ingestion of these compounds remain unknown.

Concern about potential adverse health effects of drinking water has been increased by the recent scientific recognition of mutagenic activity exhibited by the uncharacterized organic compounds (Loper, 1980). Direct support for the hypothesis that mutagenic chlorination by-products result from the reaction of chlorine with humic materials present in source water has been documented by recent findings from this laboratory (Bull et al., 1982; Meier et al., 1983). It has been shown that both fulvic and humic acids isolated from natural lakes as well as a commercial humic material react with chlorine to form direct-acting mutagenic products. The present study provides the first reported research findings on the subchronic toxicology of chlorinated and nonchlorinated humic acids. Part of these findings has been presented in abstract form (Condie et al., 1983).

METHODS

Preparation and Chlorination of Humic Acid

Solutions of commercially available humic acid (Fluka, Switzerland) were prepared on a monthly basis by a previously described method (Meier et al., 1983). The nonchlorinated samples (non-Cl) were either diluted with distilled water to yield a final total organic carbon (TOC) concentration of 1.0 g/l or chlorinated for 90 h, using a 1:1 ratio of chlorine equivalents per mole of organic carbon (final pH 2.8) (Meier et al., 1983). The TOC of the chlorinated humic acid solution was adjusted to 1.0 g/l with distilled water (1.0 Cl humic solution). The non-Cl humic samples were adjusted to pH 3.0 by the addition of HCl in order to maintain the sterility of the material. Microbial contamination was not a problem with the 1.0 Cl humic solution. The solutions were stored in teflon capped glass bottles at 4° C in the dark until use, at which time the 1.0 Cl humic solution was diluted 10-fold and 2-fold with distilled water to yield the 0.1 Cl humic and 0.5 Cl humic test solutions, (pH 2.9 and 3.5, respectively). Preliminary quality control studies were conducted to determine the stability of the Cl humic acid solutions. Total organic carbon and total organic chlorine levels were constant and mutagenic

activity was stable, which indicated that the solutions did not appear to deteriorate under the storage conditions employed. The pH of the distilled-water group was about 7.0. The distilled-water group of animals served as the control for the acidic conditions of the other exposure groups.

Animal Handling and Exposure

Male Sprague-Dawley rats (250 g) were purchased from Charles River (Portage, Mich.), held for a 1-wk quarantine period, and randomly housed, 2 per plastic cage, with hardwood chip bedding (Absorb-Dri, St. Regis Paper, Garfield, N.J.). The rats were individually marked by toe clipping. Purina Lab Chow and distilled water and chlorinated or nonchlorinated humic acid solutions were available ad libitum. The animal room was maintained at 20-22° C and 40-60% relative humidity and had 12-h interval light-dark cycles.

Eighty rats were randomly assigned to five equal exposure groups—distilled water, nonchlorinated humic acid (TOC 1.0 g/l), and three chlorinated humic groups (TOC 0.1, 0.5, 1.0 g/l). The start of the exposure of the rats to the different drinking solutions was staggered so that a maximum of 20 rats were sacrificed on any experimental day (4 rats from each treatment group). Animals were observed daily for overt signs of toxicity. Individual body weights were recorded at the start of the experiment, weekly, and just prior to sacrifice. Feed and drinking solutions were weighed and changed twice weekly.

Biological Observations

At approximately 30 d of the experiment and 1 wk prior to the 90-d sacrifice, rats were placed in metabolism cages and urine was collected overnight. Low-density mineral oil (0.5 ml) was placed in the collection vessels to prevent evaporation. Rat blood samples collected at interim periods were obtained via tail vein resection and by using a rodent bleeding carousel. At sacrifice, rats were anesthetized with sodium pentobarbital and dissected. After blood collection by cardiac puncture, gross pathological examination was performed, which was followed by removal and weighing of the liver, spleen, testes, heart, kidneys, and bladder. One kidney and the previously mentioned organs were placed in 10% buffered formalin. Hematoxylin- and eosin-stained slides were prepared from the urinary bladder and renal tissues from each animal. The slides were evaluated "blind" by a board-certified veterinary pathologist.

Cortical slices were prepared from the remaining kidney, and *p*-aminohippuric acid (PAH) active uptake into renal cortical slices was measured by the method of Cross and Taggart (1950). Lactic acid (10 *m*M) was added to the incubation medium to maximally stimulate slice uptake of PAH. The kidneys were subsequently dissected under a stereoscopic dissecting microscope to identify crystalline inclusions in the renal pelvis.

Fresh blood smears were prepared; sera from clotted blood and EDTA anticoagulated blood samples were used for clinical chemistries and hematological determinations, respectively. Hematological parameters were determined by using the Coulter ZBI-6 cell counter equipped with channelyzer and hemoglobinometer (Coulter, Hialeah, Fla.). The Centrifichem-400 fast kinetic analyzer (Union Carbide, Tarrytown, N.Y.) was utilized for clinical chemistry and serum enzyme analyses. Reagents were supplied by the respective instrument manufacturers. For quality control and calibration purposes, Coulter normal and abnormal 4-C controls and Dade I and II human controls were employed. Urine samples were examined microscopically and chemical urinalyses were performed with Ames Multistix test strips (Elkhart, Ind.). Urinary sediments were stained with Clay Adams Sedistain (Becton-Dickinson, Parsippany, N.J.).

Because of the equivocal histopathological findings from the first subchronic study, an additional 90-d study was conducted with 36 rats in order to more thoroughly examine the entire urinary tract of the rats. Male rats were randomly assigned to 3 exposure groups—distilled water, non-Cl humic, and 1.0 Cl humic groups. Similar urine specimens were collected and analyzed as described for the first subchronic study. All gross lesions involving the entire urinary tract were recorded. Tissues were preserved in Carnoy's fixative. Both kidneys, both ureters, the urinary bladder, and the urethra were processed for each animal, stained with hematoxylin and eosin, and examined microscopically by a board-certified veterinary pathologist, who was a different individual from the first subchronic study.

Statistical Analysis

All experimental data were entered into and analyzed by the Statistical Analyses System (SAS Institute, Cary, N.C.). One-way analysis of variance (ANOVA) for many of the experimental observations was conducted to test the hypothesis that there were treatment-related effects between the groups. When the ANOVA indicated a dose-related effect, Duncan's procedure was used to detect differences between treatment groups. Average weekly body weight gains and average daily food and fluid consumption data were evaluated by a repeated measures analysis of variance (Biomedical Data Processing, Berkeley, Calif.) and Duncan's procedure. Fisher's exact test for 2×2 tables was used to compare the incidence of hematuria between exposure groups. The p value of 0.05 was used as the criterion for significant differences between groups (Winer, 1971).

RESULTS

All animals survived the 90-d exposure to chlorinated and nonchlorinated humic acid except for one rat in the 0.5 Cl humic group. This rat was necropsied, and it was determined that death was not treatment-related.

TABLE 1. Body and Organ Weights of Sprague-Dawley Rats Exposed to Chlorinated and Nonchlorinated Humic Acid for 90 D[a]

Parameters	Control	0.1 Cl humic	0.5 Cl humic	1.0 Cl humic	Non-Cl humic
Body weight (g)	545 ± 12[b]	543 ± 16[b]	523 ± 10[b,c]	504 ± 12[c]	528 ± 10[b,c]
Body weight gain (g/wk)	16.4[b]	16.2[b]	14.8[b,c]	13.3[c]	15.1[b,c]
Liver (g)	19.8 ± 0.7[b,c]	20.6 ± 0.8[b]	18.7 ± 0.5[b,c]	18.7 ± 0.8[b,c]	17.9 ± 0.5[c]
Liver (% body weight)	3.6 ± 0.07[b]	3.8 ± 0.09[b]	3.6 ± 0.05[b,c]	3.7 ± 0.07[b]	3.4 ± 0.09[c]
Spleen (g)	0.65 ± 0.06	0.74 ± 0.03	0.70 ± 0.03	0.75 ± 0.03	0.76 ± 0.04
Spleen (% body weight)	0.14 ± 0.006	0.14 ± 0.006	0.13 ± 0.006	0.15 ± 0.007	0.14 ± 0.006
Testes (g)	3.4 ± 0.1	3.3 ± 0.1	3.3 ± 0.7	3.4 ± 0.2	3.3 ± 0.6
Testes (% body weight)	0.62 ± 0.02[b]	0.62 ± 0.02[b]	0.64 ± 0.02[b,c]	0.68 ± 0.02[c]	0.64 ± 0.02[b,c]
Heart (g)	1.4 ± 0.03	1.4 ± 0.04	1.5 ± 0.06	1.5 ± 0.04	1.5 ± 0.5
Heart (% body weight)	0.26 ± 0.006[b]	0.26 ± 0.005[b]	0.28 ± 0.008[c]	0.29 ± 0.004[c]	0.27 ± 0.07[b,c]
Kidney (g)	3.5 ± 0.1[b]	3.7 ± 0.1[b,c]	3.8 ± 0.1[b,c]	3.9 ± 0.1[c]	3.7 ± 0.01[b,c]
Kidney (% body weight)	0.65 ± 0.01[b]	0.68 ± 0.02[b,c]	0.73 ± 0.01[d,e]	0.77 ± 0.02[d]	0.70 ± 0.02[c,e]
Bladder (g)	0.16 ± 0.02	0.15 ± 0.02	0.13 ± 0.01	0.15 ± 0.02	0.13 ± 0.02
Bladder (% body weight)	0.029 ± 0.003	0.028 ± 0.003	0.026 ± 0.003	0.030 ± 0.003	0.025 ± 0.004

[a] Data represent the mean ± SEM for at least 15 animals from each dosage group, except for body weight gain, which represents the average weekly body weight gain from a repeated measures analysis.

[b-e] Means with the same superscript are not significantly different ($p > 0.05$).

TABLE 2. Food and Fluid Consumption from 90-D Study[a]

Dose group	Fluid consumption (g/d)	Food consumption g/d)
Control	41.9[b]	28.4
0.1 Cl humic	41.4[b]	28.5
0.5 Cl humic	34.7[c]	27.2
1.0 Cl humic	35.2[c]	26.6
Non-Cl humic	36.2[c]	28.0

[a] Data represent the daily average fluid and food consumption per animal.

[b,c] Averages with the same superscript are not significantly different ($p > 0.05$) as determined by a repeated measures analysis of variance and a multiple comparison test.

As seen in Table 1, the average body weight gain and the terminal body weight were significantly decreased in the high-dose group when compared to the distilled-water control and 0.1 Cl humic animal groups. The average body weight gain and terminal body weight in the 0.5 Cl humic and non-Cl humic groups were slightly depressed. Terminal organ weights are also summarized in Table 1. The only organ weight that is significantly increased from the corresponding distilled water control value is the average kidney weight of the 1.0 Cl humic group. Various significant differences were detected in the organ-to-body-weight ratios of the liver, testes, heart, and kidneys, which are primarily due to the differences in the total body weight between the groups.

The fluid and food consumption for the 90-d exposure period were analyzed for treatment-related differences using a repeated measures analysis of variance. No differences were found between groups for food consumption (Table 2). As compared to distilled water control values, the fluid consumption was significantly decreased in the non-Cl humic, 1.0 Cl humic, and 0.5 Cl humic groups by 14, 16, and 17%, respectively.

There were no significant differences between groups for hematological parameters (Table 3). Although there were statistically significant difference in some of the clinical chemistry values, the changes were slight and were within the normal distribution range, as described in Table 4.

The microscopic observations and chemical analyses of the urine collected at approximately 30 and 90 d of the subchronic exposure were unremarkable except for the presence of hematuria and the detection of renal pelvic crystals in rats in various groups (Table 5). The hematuria was most pronounced in the 1.0 Cl humic group at both 30 and 90 d. The incidence and severity of hematuria was greater at 90 d when compared to the 30-d period. The incidence of renal pelvic crystals was greatest in the 1.0 Cl humic group of rats. The mean volume ± SEM of urine that was collected overnight during the second subchronic study is as follows: 25.2 ± 1.8 ml, distilled water group; 20.8 ± 2.3 ml, non-Cl humic group; and

TABLE 3. Selected Hematology Parameters for Sprague-Dawley Rats Exposed to Chlorinated and Nonchlorinated Humic Acid for 90 D[a]

Parameters	Control	0.1 Cl humic	0.5 Cl humic	1.0 Cl humic	Non-Cl humic
Hemoglobin (g%)	14.2 ± 0.1	14.3 ± 0.2	14.1 ± 0.1	14.1 ± 0.2	14.1 ± 0.2
Erythrocytes (10^6/mm^3)	7.4 ± 0.1	7.2 ± 0.1	7.2 ± 0.1	7.3 ± 0.1	7.2 ± 0.1
Hematocrit (%)	39.5 ± 0.4	39.3±0.6	38.5 ± 0.6	39.2 ± 0.6	38.7 ± 0.5
Mean corpuscular volume (μm^3)	53.9 ± 0.3	54.4 ± 0.4	53.9 ± 0.5	54.5 ± 0.6	53.8 ± 0.4
Serum iron (μmol/l)	215 ± 9	221 ± 10	212 ± 5	212 ± 10	204 ± 12

[a] Data represent the mean ± SEM for at least 15 animals from each dosage group. There were no statistically significant differences between any of the treatment groups in any of the parameters listed.

TABLE 4. Clinical and Biochemical Values for Sprague-Dawley Rats Exposed to Chlorinated and Nonchlorinated Humic Acid for 90 D[a]

Parameters	Control	0.1 Cl humic	0.5 Cl humic	1.0 Cl humic	Non-Cl humic
BUN (mg %)	21.5 ± 0.6[b,c]	21.4 ± 0.5[b,c]	21.0 ± 0.4[c]	23.1 ± 0.6[b]	20.9 ± 0.8[c]
AST (IU/l)	114 ± 11	121 ± 9	129 ± 6	109 ± 6	119 ± 11
ALT (IU/l)	35 ± 5	39 ± 7	45 ± 6	31 ± 3	44 ± 5
LDH (IU/l)	1330 ± 165	1280 ± 115	1520 ± 98	1250 ± 117	1270 ± 138
ALKP (IU/l)	115 ± 7	123 ± 11	112 ± 6	112 ± 8	117 ± 9
Creatinine (mg %)	0.64 ± 0.02[b,c]	0.68 ± 0.03[b,c]	0.67 ± 0.02[b,c]	0.70 ± 0.02[b]	0.61 ± 0.04[c]
Protein (g %)	5.8 ± 0.1	5.8 ± 0.1	5.5 ± 0.1	5.8 ± 0.1	5.7 ± 0.1
Albumin (g %)	3.5 ± 0.1	3.5 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1
PAH uptake (slice/ medium ratio)	20.5 ± 1.4	18.8 ± 0.8	18.6 ± 1.8	19.8 ± 1.7	19.7 ± 1.0

[a]Data represent the mean ±SEM for at least 15 animals from each dosage group. Abbreviations: BUN=blood urea nitrogen, AST=aspartate aminotransferase, ALT=alanine aminotransferase, LDH=lactic dehydrogenase, ALKP=alkaline phosphatase, PAH=*p*-Aminohippurate.

[b]Means with the same superscript are not significantly different ($p > 0.05$).

23.7 ± 1.8 ml, 1.0 Cl humic group (no statistically significant differences).

In order to better understand the etiology of the hematuria, a thorough histopathological evaluation was performed on the kidneys and urinary bladders from rats killed following the first 90-d exposure study to chlorinated and non-chlorinated humic acid and from an earlier

TABLE 5. Incidence of Hematuria in Sprague-Dawley Rats Exposed to Chlorinated and Nonchlorinated Humic Acid[a,b]

Duration of exposure	Severity of hematuria[c]	Control[d]	0.1 Cl humic	0.5 Cl humic	1.0 Cl humic[d,c]	Non-Cl humic[e]
30 Days	0	16	15	13	11	15
	1	0	0	1	2	0
	2	0	1	1	0	0
	3	0	0	1	3	0
	4	0	0	0	0	1
90 Days	0	12	10	11	6	12
	1	4(1)	4	3	5(1)	2(1)
	2	0	0	0	1(1)	0
	3	0	1(1)	0	3(2)	1
	4	0	0	1	1	1(1)

[a]Comparisons between all groups were made by Fisher's exact test for 2 × 2 tables. Only the presence or absence of hematuria was evaluated. The incidences of trace hematuria were included in the statistical evaluation of the data.

[b]The number of rats which were observed at necropsy to have crystals in the renal pelvis is indicated in parentheses.

[c]Key to severity ratings: 0 = undetected (>50 μg/dl hemoglobin), 1 = trace, 2 = slight, 3 = moderate, 4 = moderately severe/severe.

[d]Significant difference between two groups at both 30 and 90 d ($p < 0.05$).

[e]Significant difference between two groups at only 90 d ($p < 0.05$).

30-d study of rats exposed to similar drinking solutions. The histopathological findings are briefly summarized as follows. A few changes, such as chronic inflammation and epithelial hyperplasia, were observed in the urinary bladders of these rats; however, the changes detected in the urinary bladders were not unusual for Sprague-Dawley rats and were interpreted as spontaneous incidental lesions. Renal changes including nephropathy, chronic interstitial inflammation, and pyeloonephritis were observed in each group and did not appear to be treatment-related, since these nonspecific findings are commonly seen in laboratory rodents of all types. A finding incidental to the experiment was an increased incidence of nephropathy and its severity in the 90-d rats as compared to the 30-d animals. The histopathological findings were equivocal for explaining the etiology of the increased incidence and severity of hematuria observed in the 1.0 Cl humic group because the pelvic crystals observed at necropsy dissolved in the formalin tissue fixative since they appear to be water-soluble.

A second 90-d study was conducted in which the complete urinary tract including ureter and urethra were trimmed and examined microscopically. The tissues were placed in Carnoy's fixative in order to prevent the crystals from dissolving. Nonspecific changes such as chronic renal interstitial inflammation were observed in all treatment groups. Renal pelvic crystals were found in rats with moderate to severe incidences of hematuria. In fact, there was a 100% correlation between renal crystal formation and hematuria with the rats having the more severe cases of hematuria.

DISCUSSION

Humic substances, the major component of the organic material found in drinking water, have a diverse structure which is not homogeneous. It is not surprising that drinking water disinfectants can react with humic material to produce a diverse group of by-products. A key issue that our laboratory is currently addressing is whether chemicals produced through disinfection of drinking water have significant biological activity. Our laboratory has published research findings that the chlorination of humic substance yields mutagenic reaction by-products and that chlorination of commercial humic acids can serve as a model for the examination of mutagen formation during water chlorination (Bull et al., 1982; Meier et al., 1983). Since human health risk assessment cannot be estimated solely by in vitro test results, subchronic exposures were conducted with chlorinated and nonchlorinated humic acid solutions. The design of the first 90-d study and selection of biological endpoints to be measured were determined by the results of an earlier 30-d study in which the detection of hematuria was the primary toxicological observation.

The most significant finding of this study was the observation of

increased incidence and severity of hematuria in the 1.0 Cl humic group. This finding confirms what was detected in an earlier 30-d experiment. Experimentation to date indicates that a possible cause of some of the incidences of hematuria was pelvic crystal formation. In fact, hematuria is almost always present as a clinical manifestation of hephrolithiasis in humans (Epstein, 1974). However, renal crystals were not detected in all rats having moderate to severe cases of hematuria and were present in several rats with only trace amounts of hemaglobin in the urine. The renal crystal particles (size <0.5 mm) were surrounded by a mucous coating, yet their crystallinity could be clearly demonstrated by crushing and observing refractile crystal fragments under high power magnification. Unfortunately, the quantity of these materials isolated was insufficient for infrared (IR) spectroscopy, and their presence during the subsequent histopathological sectioning was confirmed following subchronic exposure. Light microscope histopathology indicated the presence of irritation which was suggested by dilated pelvic cavities with associated hyperplastic changes of the transitional epithelium. Other observable structural changes in renal tissue such as nephropathy, interstitial inflammation, and pyelonephritis did not appear to be treatment-related, since the changes were randomly distributed among the treatment groups. Clinical chemistry parameters that may indicate renal or other systemic damage were not altered.

It is possible that the effects that were observed were caused by dehydration and the acidity of the drinking solutions. However, clinical observations and determinations did not indicate that the rats were dehydrated. The urine volumes that were collected overnight were not statistically different when comparing treatment gorups with the distilled water group. Since there were no detected differences between the distilled-water control group of rats and three treatment groups that received acidic drinking solutions (0.1 Cl humic, 0.5 Cl humic, and non-Cl humic groups), it is felt that the hematuria observed in the 1.0 Cl humic group was due to precipitation of chlorination by-products of humic acid rather than the acidic drinking-solution conditions.

Since the distilled water control animals showed evidence of minimal renal pelvic hemorrhage, the authors' view is that the trace levels of hematuria could be due to the early onset of kidney pathology manifested by the Sprague-Dawley rats. The existing predisposition in the Sprague-Dawley rats for developing idiopathic renal changes could therefore be accelerated in the 1.0 Cl humic treatment group.

Relative risks to human health from the various methods of drinking water disinfection cannot be completely estimated at this time. This study was designed to determine if chlorination of humic acid material, which is found in finished drinking water supplies, presents a potential health problem. These studies suggest that there is minimal risk from exposure to chlorination by-products of humic acids as far as target organ effects are concerned. The highest no observable effect level for TOC in this study was 0.5 g/l, which represents a safety factor to the human popula-

tion of least 100-fold because 95% of the municipal drinking-water systems tested in a national survey have TOC levels less than 5 mg/l (Symons et al., 1975). Other studies are currently underway in our laboratory to better understand the relative hazards of drinking water chlorination and its various alternatives (chlorine dioxide, chloramines, ozone).

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